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(54) Title: IMMUNOGENIC ANTIGEN-CARRIER PROTEIN CONJUGATE FOR USE IN A VACCINE AGAINST MALARIA

(57) Abstract

A conjugate comprising a synthetic immunogenic peptide, which hasan amino acid sequence corresponding to that of an immunodominant epitope of the circumsporozoite protein of the malaria parasite *P. falciparum* and a carrier protein selected from the group consisting of carrier proteins used in vaccine preparations.

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IMMUNOGENIC ANTIGEN-CARRIER PROTEIN CONJUGATE FOR USE IN A VACCINE AGAINST MALARIA

The United States government has rights in this invention by virtue of Grants No. DPE-0453-C-00-2002-00 from the Department of State, Agency for International Development and 5R01-AI-17429-03 from the Department of Health and Human Services.

The present application incorporates by reference the entire disclosures of:

(a) U.S. Patent No. 4,466,917 of Nussenzweig, R., et al, issued on August 21, 1984 and entitled Malaria Vaccine;

(b) Assignee's co-pending U.S. Patent Application Serial No. 574,553 of Ellis, J. et al, filed on January 27, 1984 and entitled "Protective Peptide Antigen";

(c) Assignee's co-pending U.S. Patent Application Serial No. 633,147 of Ellis, J. et al, filed on July 23, 1984 and entitled "Protective Peptide Antigen Corresponding to Plasmodium falciparum Circumsporozoite Protein."

(d) Assignee's co-pending U.S. Patent Application Serial No. 649,903 of Vergara, U. et al, filed on October 26, 1984 and entitled "Cross-Reactive and Protective Epitopes of Circumsporozoite Proteins"; and

(e) Assignee's co-pending U.S. Patent Application Serial No. 695,257 of Nussenzweig, V. et al, filed on January 28, 1985 and entitled "Immunodominant Epitope of the Circumsporozoite Surface Protein."

Background of the Invention

The present invention relates to conjugates of an antigen and a carrier protein useful for providing protective immunity against malaria. More particularly, the present invention relates to conjugates of a peptide and a carrier protein useful for providing protective immunity against the sporozoite stage of malaria.

It is known that inoculation of relatively small amounts of X-irradiated sporozoites into rodents, primates and humans results in protective immunity. The immunity is stage-specific (i.e. it protects against the sporozoite stage of malaria but not against the blood stages) and in most instances species-specific (i.e. inoculation with sporozoites of a single species usually confers immunity only against that species) but not strain-specific (innoculation with sporozoites of one species originating from one particular endemic area confers immunity against sporozoites of the same species originating from other endemic areas).

It is also known that incubation of every species of sporozoites with antisera from any host immunized with X-irradiated sporozoites of the same species results in the formation of tail-like precipitate on the sporozoite surface. (This reaction is known as "circumsporozoite precipitation reacton" or "CSP reaction.") It also results in complete neutralization (loss) of sporozoite infectivity.

The target antigens of these anti-sporozoite antisera have been identified by monoclonal antibodies. They belong to a family of polypeptides (circumsporozoite surface- or CS-proteins) that normally cover the entire surface membrane of the sporozoite but are shed upon reaction (cross-linking) with antibodies.

All known CS proteins contain strongly immunodominant repeated epitopes. Monoclonal antibodies to these epitopes neutralize sporozoite infectivity both *in vitro* and *in viv* .

The gene corresponding to the CS protein of P. falciparum, a human malaria species, has been cloned. The immunodominant epitope comprises the sequence H-(Asn-Ala-Asn-Pro)$_3$-OH -- also designated as (NANP)$_3$. This epitope is found in Plasmodium falciparum strains from all endemic areas of the world, and is represented many times in the CS molecule. (Enea, et al. 1984. Science: 225, 628; Dame, et al. 1984, Science: 225, 593; Zavala, F. et al., Fed. Proc. 43: 1808, 1984 and J. Immunol, in press).

The ultimate goal of all this research is to develop a preventive vaccine against malaria.

Such a vaccine should not only be effective in conferring (or boosting) immunity, but should be easy and inexpensive to produce in a mass scale, in view of the great number of persons in need of immunization.

A malaria vaccine using synthetic short length peptides as an immunization agent would be advantageous over another vaccine that used whole CS proteins as the immunization agents because of ease of manufacture, lower cost and large supply of immunogen.

Summary of the Invention

It has now been discovered that conjugates of a peptide comprising the immunodominant epitope of P.falciparum CS protein and a carrier are effective in raising high titers of antibodies in vivo. These antibodies recognize sporozoites and neutralize sporozoite infectivity in vitro by a vigorous CSP reaction. The preferred peptide is H-(Asn-Ala-Asn-Pro)$_3$-OH -- also designated (NANP)$_3$ -- i.e., a dodecapeptide consisting of the amino acid sequence NANP tandemly repeated three times, and the preferred carrier is tetanus toxoid.

It has also been discovered that most or all antibodies in human sera from endemic areas recognize a synthetic peptide, (NANP)$_3$. This further supports the notion that (NANP)$_3$ indeed represents faithfully the

repetitive epitope of P. falciparum CS protein. Therefore, the conjugates of the present invention are useful in the development of a protective vaccine against malaria.

The present invention is further described in detail below.

Brief Description Of The Drawing

Figs. 1-3 are plots of radioactivity counts per minute observed in immunoradiometric assays against the reciprocal serum dilution from rabbits immunized with conjugates according to the present invention.

Fig. 4 is a plot of the results of an immunoradiometric assay of rabbit antisera raised against the conjugate of the present invention in the presence of increasing concentrations of $(NANP)_3$ in the fluid phase.

Fig. 5 depicts the results of Western blotting of Plasmodium falciparum extracts revealed by rabbit antisera raised against (a) Plasmodium falciparum sporozoite extracts; (b) the conjugate of the present invention with complete Freund's adjuvant; (c) normal rabbit serum; and (d) the conjugate of the present invention in incomplete Freund's adjuvant.

Fig. 6 is a plot of the results of an immunoradiometric assay of rabbit antisera raised against a conjugate according to the present invention performed in the presence of increasing concentrations of P. falciparum and P. berghei sporozoite extracts.

Fig. 7 depicts the proportion of positive serum reactions with $(NANP)_3$ in humans from endemic areas according to the age of the human subjects.

Fig. 8 is a histogram of the result of an immunoradiometric assay of the same human sera, as those used to generate Figure 7; the assay was conducted in the presence or absence of competing $(NANP)_3$ or another peptide in the fluid phase.

Detailed Description Of The Invention

The present conjugates have been found to generate high titers of anti-*P. falciparum* sporozoite antibodies by immunization of rabbits, mice and aotus monkeys, whether these conjugates were emulsified in complete or incomplete Freund's adjuvant. The conjugates elicited anti-sporozoite antibodies in rabbits even in the absence of adjuvant.

Most of the antibodies raised against the conjugate of the present invention recognize the *P.falciparum* CS protein and neutralize sporozoite infectivity *in vitro* at low concentrations (below about 0.2 micrograms/ml).

The antibody titers increase with the amount of antigen injected.

Most antibodies to sporozoites in human sera from endemic areas react with $(NANP)_3$ and confirm that the epitope of the *P. falciparum* CS protein is not strain-specific. Accordingly, this epitope would not give rise to strain-specific antibodies.

Therefore, the present conjugate is a good candidate for developing a malaria vaccine, especially one that could be used to immunize humans in different geographical areas.

The present conjugates have been prepared by conjugation of $(NANP)_3$ with tetanus-toxoid. In addition to being a carrier protein, tetanus-toxoid is an immunization agent in its own right. However, there are many other such carriers that could by used. Examples are: diphtheria toxoid (available from many commercial sources: Lederle Laboratories, Pearl River, N.Y.; Merrell Dow Pharmaceuticals, Cincinnati, Ohio; Eli Lilly & Co., Indianapolis, Indiana et al) other proteins and polysaccharides well-known for that purpose as well as synthetic peptides and polymers comprising lysine and arginine groups. Use of these other carriers is fully expected to give rise to effective conjugates.

In some of the examples below, the conjugates have been prepared using glutaraldehyde as a coupling reagent. However, other coupling procedures are readily available, such as one using water soluble carbodiimides (J. Biol. Chem. 242 2447-2453, 1967) or bis-diazobenzidine [following addition of an extra tyrosine residue at the N-terminal of $(NANP)_3$, Proc. Nat'l Acad. Sci., 77:5197-5200, 1980] or malimidobenzoyl-N-hydroxy succinimide ester [following addition of an extra cysteine residue or other sulphydrils to the N-terminal of $(NANP)_3$; see Proc. Nat'l Acad. Sci., 78:3403-3407, 1981]. A particularly preferred embodiment of the present invention lies in the addition of a cysteine residue to the N-terminal of the peptide and the use of malimido benzoyle-N-hydroxy succinimide ester as a coupling reagent.

In the present invention, Freund's complete (as well as incomplete) adjuvant was used as an adjuvant. The function of an adjuvant is to enhance the immune response. Any adjuvant suitable for use in vaccine preparations can be used.

Although the present results indicate that the presence of an adjuvant in a vaccine preparation is not essential, an adjuvant advantageously increases the immunogenicity of a conjugate and is therefore preferably included. Other suitable adjuvants are aluminum phosphate, aluminum hydroxide, muramyl dipeptide or derivatives et al.

The present invention is described further below by reference to particularly preferred embodiments. However, as will be readily recognized by persons of ordinary skill in the art, a number of modifications, additions, and substitutions may be made without departing from the scope or spirit of the present invention as disclosed in this specification, the accompanying claims and the appended drawings.

The purpose of the following examples is to illustrate the present invention but not to limit its scope.

Example 1: Synthesis and Purification of $(NANP)_3$

The dodecapeptide $(NANP)_3$ was synthesized by the solid-phase method of Merrifield, R.B. (1962) Fed. Proc. Fed. Am. Soc. Ex. Biol., 21:412; and (1963) J. Chem. Soc., 85:2149.

The attachment of the C-terminal amino acid residue, Boc-Pro, was onto hydroxymethyl-Pam-[copoly(styrene-1% divinylbenzene)]-resin support which was synthesized from underivated polystyrene resin from Bio-Rad, Richmond, California, (as disclosed by Mitchell, A.R. et al, 1976 J. Am. Chem. Soc. 98:7357) to prevent loss of peptide chains during synthesis.

The thus prepared Boc-Pro-OCH_2-Pam-resin (2 g, 0.4 mmol substitution per gram of resin) was placed into the reaction vessel of a modified Beckman 990 synthesizer (Beckman Instruments, Palo Alto, California). Synthesis was performed using a computer, which optimized the coupling steps.

The protected dodecameric peptide-resin was deprotected batchwise (0.5 gram) by HF-anisole (9:1, v/v, 10 ml) for 60 minutes at 0°C.

The cleavage yield was 91% based on back hydrolysis of the resin by 6N HCl. The purity of the crude peptide was determined to be greater than 85% by high pressure liquid chromatography on a reverse-phase C-18 column (4.6 x 250 mm manufactured by Vydac, Hesperia, Calif.) using an aqueous CF_3CO_2H and CH_3CN gradient system as follows: eluant A contained 100 ml H_2O and 0.05 ml CH_3CO_2H, and eluant B contained 60 ml H_2O, 40 ml CH_3CN and 0.05 ml CF_3CO_2H. The system was eluted at 1 ml/min in a linear gradient of 10%B to 85 %B in 30 min in a Waters Associates, (Milford, MA.) HPLC system. Detection was at 215 nm and a major symmetrical peak was detected at 11.4 minutes. This peak accounted for more than 85% of the crude peptide content and contained the correct amino acid ratios for $(NANP)_3$.

Preparative purification (60 mg) was carried out in a low-pressure liquid chromatography system on a 2.5 x 30 cm Michel-Miller column.

The eluting system consisted of 750 ml of eluent A (712.5 ml H_2O, 37.5 ml CH_3CN and 0.375 ml of CF_3CO_2H and 800 ml of eluent B (480 ml H_2O, 320 ml CH_3CN and 0.4 ml CF_3CO_2H). The system was eluted at 1.5ml/min. Fractions were collected at 5 ml/min in a linear eluent B gradient (0-100%B) in 16 hr by an LDC pump. Detection was at 215 nm and a major symmetrical peak was detected between fractions 43 and 57. The fractions were collected, the CF_3CO_2H was neutralized by concentrate NH_4OH and CH_3CN was removed by vacuum. The aqueous portion was lyophilized.

The purified peptide gave a single symmetrical peak upon reverse phase analytical high pressure liquid chromatography. On amino acid analysis, the peptide gave Asp:Ala:Pro, 2.02:1:0.99 (theoretical value 2:1:1). The results of preparative scale were used to optimize the coupling steps in the synthesizer and to program the computer accordingly.

Example 1a: Synthesis of Ac-Cys(NANP)$_3$-OH and Cys(NANP)$_3$-OH Boc-Pro-hydroxymethyl-resin, 1

Boc-Pro-OH (20.66 g, 96 mmol) and DCC (9.89 g, 48 mmol) are reacted in DMF (400 ml) for 1 hr, filtered and the resultant preformed symmetric anhydride is added to hydroxymethyl-resin (20 g; 0.8 meq/g; 16 mmol) in the presence of 4-dimethylaminopyridine (0.586 g; 4.8 mmol). The slurry is shaken for 24 hr at room temperature. An aliquot (approx. 50 mg) is hydrolyzed with 1 ml of 1:1 propionic acid/HCl in a sealed tube at 150° for 1 hr. Amino acid analysis reveals a substitution level of 0.36 mmol/g. The resin is stirred in CH_2Cl_2:pyridine (400 ml:12.94 ml) and benzoyl chloride (18.75 ml, 160 mmol) added and stirring continued at 0° for 30 min and at room temperature for 1 hr. The

reaction mixture is filtered and washed with CH_2Cl_2 (3 x 350 ml), DMF (2 x 350 ml), CH_2Cl_2 (2 x 350 ml), MeOH (2 x 350 ml) and dried in vacuo to give 21.6 g of 3.

Pro-Hydroxymethyl-resin, 2

Boc-Pro-Hydroxymethyl-resin, 1 (21 g; 0.36 mol/g; 7.56 mmol) is washed with 600 ml of CH_2Cl_2, deprotected with 300 ml of 50% TFA-CH_2Cl_2 for 1 min, washed with 300 mL of CH_2Cl_2 and deprotected again with 300 ml of 50% TFA-CH_2Cl_2 for 20 min. The reaction mixture is washed 4 times with 300 ml of CH_2Cl_2 and neutralized by washing 2 times with 300 ml of 8% DIEA-CH_2Cl_2 (5 min each), 2 times with 300 ml of CH_2Cl_2, 2 times with 300 ml of 2-propanol and 6 times with 300 ml of CH_2Cl_2.

Boc-Asn-Pro-Hydroxymethyl-resin, 3

Boc-Asn-OH (7.02 g, 30.24 mmol, 4.0 equiv.) is added to Prohydroxymethyl-resin (2, 7.56 mmol) in 300 ml of CH_2Cl_2 and agitated for 5 min. Dicyclohexylcarbodiimide (6.23 g, 30.24 mmol, 4.0 equiv.) is added and agitation proceeds for 60 min. Diisopropylethylamine (3 ml) is added (1% by volume) and agitation continued for an additional 15 min. The reaction mixture is filtered and washed 3 times with 300 ml CH_2Cl_2. An aliquot of resin (approx. 1.5 mg) is removed and monitored by the Ninhydrin Reaction as follows: The peptide-resin is placed in a small test tube and treated with 3 drops each of solutions A, B and C [Solution A: 500 mg ninhydrin in 10 ml of EtOH; Solution B: 80 g phenol in 20 ml EtOH; Solution C: 2 ml 0.001M KCN in 100 ml pyridine]. The tube is heated at 95-100° for 5 min and the beads and solution are examined visually. The peptide coupling reaction is determined to be incomplete if the Ninhydrin Reaction is positive and gives either a blue solution or blue beads. If the Ninhydrin Reaction is positive the entire coupling cycle is repeated.

Ac-Cys(Dmb)-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-hydroxymethyl-resin, 4

The Boc-Asn-Pro-hydroxymethyl-resin, 3 (7.56 mmol) is charged into a 1000 mL reaction vessel, attached to a

Kraft Shaker and subjected to the washing, deprotection and neutralization procedures specified in Step 2. Coupling reactions and washings are then carried out with 30.24 mmol (4 equiv.) of Boc-amino acids by the DDC procedure specified in Step 3 (or *via* HOBt-ester) in the sequence shown:

Coupling

No.	Residue	Acid	Amounts	Coupling Procedure
1	11	Ala	11.5 g	DCC
2	10	Asn	35.0 g	HOBt-Ester
3	9	Pro	22.0 g	DCC
4	8	Asn	21.0 g	HOBt-Ester
5	7	Ala	11.5 g	DCC
6	6	Asn	28.0 g	HOBt-Ester
7	5	Pro	19.5 g	DCC
8	4	Asn	35.0 g	HOBt-Ester
9	3	Ala	17.2 g	DCC
10	2	Asn	35.0 g	HOBt-Ester
11	1	Cys(Dmb)	31.0 g	DCC

A modified protocol is used for the 1-hydroxybenzotriazole (HOBt)-dicyclohexylcarbodiimide (DCC) coupling procedure. In these cases the Boc-amino acids (4 equiv.) dissolved in 300 ml of DMF, in a separate flask, and reacted with 1-hydroxybenzotriazole (5.09 g, 33.26 mmol, 4.4 equiv.) for 45 min. The reaction mixture is filtered (to remove dicyclohexylurea) and added to the peptide resin [which was washed with 300 ml of DMF prior to, and subsequent to, the addition of the HOBt-ester] and agitated for 1 hr. The washing cycles are otherwise identical to the protocol for the DCC-coupling procedure. After the completion of the 11 coupling reactions and final deprotection with 50% TFA-CH_2Cl_2, the N^{a-} amino group of Cys is acetylated with a solution of acetic anhydride (150 ml):Pyridine (150 ml) for 1 hr.

The peptide-resin **4**, is finally washed 4 times with 300 ml of CH_2Cl_2 and dried *in vacuo* to give 20.5 g of **4**.

Ac-Cys-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-OH, Ac-Cys(NANP)$_3$-OH, 5

A portion of peptide-resin 4 (10.5 g) is placed in an HF-reaction vessel and 10.5 ml of dithioethane is added. Liquid HF (94.5 ml) is condensed into the reaction vessel and stirring proceeds at 0° for 1 hr. Following evaporation to dryness *in vacuo* the residue is treated with 500 mL of EtOAc and filtered. The precipitate is extracted 4 times with 80 ml each of TFA and evaporated. The oily residue is triturated 4 times with 300 mL of anhydrous ether and dried *in vacuo* to give 1.98 g of crude 8.

The 1.98 g of crude 5 is dissolved in 30 ml of H_2O (containing 0.1% TFA), filtered through an 0.8 micron Type AA Millipore filter and refiltered through a 0.45 micron Type HA Millipore filter. The filtrate (total volume, 45 ml) is charged onto a Nucleosil C_{18} reversed-phase column (2.54 x 25 cm) [previously equilibrated with 5% acetonitrile (containing 0.1% TFA)-H_2O (containing 0.1% TFA)]. The column is eluted (flow rate, 5 ml/min) with a solvent system consisting of acetonitrile (containing 0.1% TFA) - water (containing 0.1% TFA) in a linear gradient mode from 5% acetonitrile to 25% acetonitrile in 120 min using an LDC Constametric IIG with a Gradient Master and Spectromonitor III Detector and LKB Fraction Collector. [Settings: wavelength: 215 nm; recorder speed: 1 mm/min; sensitivity: 2.0 AUFS; fractions: 1 min (5 ml)/fraction.

Aliquots are analyzed by HPLC using an LDC Constametric IIG equipped with a Gradient Master Spectromonitor III Detector and Micromeritics 725 Autoinjector. [Settings: wavelength: 206 nm; column: Lichrosorb RP-8 (5 micron); eluant: acetonitrile - 0.1M $HClO_4$ (pH 2.5); gradient: linear, 8% acetonitrile to 20% acetonitrile in 20 min; sensitivity: 0.2 AUFS]. The product emerges in fractions 55 to 65 which are combined, evaporated and lyophilized to give 577 mg of pure product. Side-bands (fractions 50-54 and 66-70, 421 mg) are also obtained. Yield:

914 mg (19.6%). Amino Acid Anal. (6M HCl; 150°, 1 h): Asp, 5.85; Pro, 3.02; Ala, 3.11; Cys, 1.13. $[\delta]_D^{25}$-163.9]° (C 0.86, 0.2N AcOH). (+) FAB Mass Spectroscopy (6 KV): Calcd. for $C_{53}H_{81}N_{19}O_{20}S$, 1352.56; Found, 1352. ^{1}H-NMR Spectroscopy (DMSO-d_6): 1.18 (3H,d, J=7Hz, CH_3 of Ala), 1.22 (6H,d,J=7Hz, 2 x CH_3 of Ala), 1.89 (3H,s,NAc). Analytical HPLC: Column, Lichrosorb RP-8 (5 micron); Eluant, (A) 0.1M $HClO_4$ (pH 2.5) - (B) Acetonitrile in a linear gradient mode from 5%(B) to 15%(B) in 30 min; Detection, 206 nm; Purity estimated to be greater than 95% (retention time: 23 min).

H-Cys-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-OH, Cys(NANP)$_3$-OH, 6

A 1 g portion of peptide-resin (from 4) which was deprotected with 50% TFA-CH_2Cl_2 but not N^a-acetylated, was subjected to an HF cleavage and work-up as in 5. Yield: 192 mg.

The crude product was subjected to purification by HPLC as in 5. The mixture was loaded onto a Nucleosil C_{18} reversed phase column (2.54 x 25 cm) and the column eluted (flow rate = 5 ml/min) with a solvent system consisting of acetonitrile (containing 0.1% TFA)-water (containing 0.1% TFA) in a linear gradient mode from 7% acetonitrile to 22% acetonitrile in 180 min. The monitoring of the purification was exactly as in 5. The product emerged in fractions 86-93 which were pooled, evaporated and lyophilized to give 61.2 mg (0.047 mmol, 12.7%) of Cys(NANP)$_3$-OH. Sidebands (fractions 84-85 and 94-97, 19.2 mg) were also obtained. Total yield: 15.5%.

The product was shown to be homogeneous in the described analytical HPLC system (retention time = 18 min) and gave the expected amino acid composition (6N HCl-TGA; 110°; 24 h): Asp, 5.96; Pro, 3.13; Ala, 3.07.

Example 2: Antigen-Carrier Conjugation

Fluid tetanus toxoid (TT) supplied by the Pasteur Institute, Paris, France, was dialyzed against distilled

water for 48 hours and lyophilized. Partially purified tetanus toxoid suitable for use in humans for vaccination is also commercially available from Burroughts Wellcome Research Triangle Park, N.C., or from Wyeth Laboratories, Dir. of Am. Home Products Corp., Philadelphia, Penn.

Equal volumes of TT (1 mg/ml) and $(NANP)_3$ (1 mg/ml) were mixed; a solution 0.37% glutaraldehyde in water was added to a final concedntration of 0.02%. After incubation for six hours at room temperature, the mixture was extensively dialyzed against distilled water for 48 hours and lyophilized. The polymerized toxoid and peptide recovery ranged between 68 and 80% by weight. By HPLC analysis, the preparation contained less than 1% of free peptide.

The resulting material was resuspended in 2 ml of phosphate buffered saline (pH 7.4) and kept in the refrigerator.

Two lots of antigen were thus prepared and used to immunize groups of rabbits with 1.0 or 0.1 mg protein in the presence or absence of Freund's adjuvant -- complete or incomplete.

Example 3: Immunization

Rabbits (2-2.5 kg) were injected in one hind foot pad and opposite thigh intramuscularly with a total of 2 ml of the vaccine preparation of Example 2, either emulsified in Freund's adjuvant (both complete and incomplete) or diluted in PBS. The total amount of injected protein was 1 mg or 0.1 mg per rabbit. When adjuvant was not used, a booster of the same dose of vaccine was given subcutaneoulsy two weeks after the first injection. The adjuvant mixture was prepared by emulsifying equal volumes of the vaccine described in Example 2 (at a concentration of 2 mg/ ml) in the adjuvant.

The rabbits were bled four weeks after immunization.

Example 4: Solid-Phase Immunoradiometric (IRMA) Assays

Flexible polyvinylchloride microtiter plates (Dynatech Laboratories, Inc., Alexandria, Virginia) were incubated with 150 microliters of 50 micrograms/ml bovine serum albumin (radio-immunoassay grade, Sigma Chemical Co., Inc., St. Louis, Mo.) for four hours at 37°C. After washing several times with phosphate-buffered saline (pH 7.4), 20 microliters of a solution containing 100 micrograms per ml of $(NANP)_3$ and 0.25% glutaraldehyde by volume were placed in each well and incubated at room temperature for two hours. The wells were washed three times with PBS and incubated overnight with 150 microliters of PBS containing 1% bovine serum albumin (BSA) and 0.5M ethanolamine at pH 7.5 (PBS-BSA-Eth buffer).

Rabbit serum samples (obtained four weeks after injection) were diluted in buffer PBS-BSA-Eth containing 0.5% Tween-20, and 30 microliters were placed in each well. After incubation for one hour at room temperature, each well was washed three times with PBS-BSA-Eth containing 0.5% Tween-20 (ICI Americas, Inc., Wilmington, Delaware) to eliminate unbound material.

Thirty microliters (7×10^4 counts per minute) of ^{125}[I]-labeled, affinity-purified, goat anti-rabbit immunoglobulin (Miles-Yeda, Elkart, Ind.) were placed in each well to label the bound antisera. The wells were washed, cut and counted.

The results of the immunoradiometric assays are shown in Figures 1-3.

In Fig. 1, antibody titers (defined as the serum dilution giving 10^3cpm in the IRMA) between 1,000 and 10,000 were found in six rabbits immunized with 1 mg of antigen [$(NANP)_3$-TT] in incomplete Freund's adjuvant (single dose). Both lots of conjugate vaccine appear to be equally effective. Antisera from lot 1 rabbits are represented by white circles for the first rabbit, black circles for the second rabbit and white squares for the

third rabbit. Antisera from lot 2 rabbits are represented by black triangles, white up-right triangles and white inverted triangles, respectively.

The results of immunoradiometric assays conducted with antisera obtained by immunization with $(NANP)_3$-TT in complete Freund's adjuvant were identical (not shown).

A control immunoradiometric assay using pre-immune rabbit serum gave negative results (30-50 cpm above background).

Fig. 2 shows the antibody titers obtained when 0.1 mg of antigen [$(NANP)_3$-TT] was injected in three rabbits with incomplete Freund's adjuvant. The serum titers were 320-80). Pre-immune sera were negative.

Fig. 3 shows the antibody titers obtained when 0.1 mg of antigen was injected in the absence of any Freund's adjuvant. The titers ranged between 80 and 10 cpm. Again, pre-immune sera were negative. No reactivity was observed when the plates were coated with peptide alone. The serum titers remained practically unchanged for at least 10 weeks after immunization.

The above results indicate that effective immunization can occur with $(NANP)_3$ conjugated to tetanus toxoid, preferably emulsified in an adjuvant. The quantity of antibodies produced increases with the amount of antigen injected. Antisporozoite antibodies that recognize the dodecapeptide $(NANP)_3$. were elicited even in the absence of adjuvants.

Example 5: Indirect Immunofluorescence Assay

The same rabbit sera used in the immunoradiometric assay of Example 4 were also assayed for reactivity with the surface membrane of glutaraldehyde-fixed sporozoites of P. falciparum.

The immunofluorescence assay was disclosed in Nardin, E.H., et al (1979) Science 206:597. Fixed parasite preparations were obtained by incubation with 0.1% glutaraldehyde for 10 min at room temperature. The sporozoites

were washed and resuspended to a concentration of 3-5 x 10^5/ml. The sporozoites were distributed in multiple-well slides, air dried and stored at -70°C.

The results are summarized in the inserts of Figures 1-3. The correlation between antibody titers (obtained by immunofluorescence) with those obtained in IRMA was highly significant ($p < 0.001$) by the Spearman rank correlation coefficient.

Example 6: Inhibitory Effect of $(NANP)_3$ On Immunoradiometric Assay.

The assay of Example 4 was repeated except that increasing concentrations of $(NANP)_3$ peptide were added to the antisera in the incubation mixture.

A constant (1/1,000) dilution of rabbit antisera to the present conjugate was incubated with serial dilutions of $(NANP)_3$. $(NANP)_3$ effectively inhibits the immunochemical reaction between rabbit anti-conjugate antisera and $(NANP)_3$ bound to the wells.

The results, shown in Fig. 4, demonstrate the specificity of the peptide-antiserum reaction.

Another immunoradiometric assay was performed to determine the proportion of the anti-conjugate antibodies that reacted with active CS protein.

The assay used a constant dilution of a rabbit antiserum to the conjugate (1/100) in the presence of increasing concentrations of P. falciparum sporozoite extract. As shown in Fig. 6, the reactivity of the antibody with the bound $(NANP)_3$ diminished to about 30% of control (no sporozoite extract) levels. This means that 70% of the reactivity of the anti-conjugate antibodies was absorbed by the CS protein of P. falciparum. The inhibitory effect was specific, since it was not observed with extracts of sporozoites of Plasmodium berghei.

Example 7: Western Blotting

Western blotting was used to measure the ability of the anti-conjugate antisera to react with the CS protein and its precursors.

Western blotting was performed as follows: Sporozoite extracts (10^5/ml) were subjected to electrophoresis in a 10% sodium dodecyl sulfate polyacrylamide gel. The separated proteins were electrophoretically transferred to nitrocellulose sheets (as disclosed by Towbin, H. et al., Electrophoretic Transfer of Proteins From Polyacrylamide Gels to Nitrocellulose Sheets, Proc. Nat'l. Adad. Sci. (USA) 76:4350-4354 (1979)). The nitrocellulose paper was saturated with PBS containing 5% BSA and 10% normal goat serum for 2 hours at 37°C. The various lanes were cut and each lane was incubated as follows: (1) with rabbit antiserum against whole P. falciparum extract; (2) with anti-[$(NANP)_3$-TT] (from immunization with complete Freund's adjuvant); (3) with normal (preimmune) rabbit serum; and (4) with anti-[$(NANP)_3$-TT] from immunization with incomplete Freund's adjuvant. The antiserum against whole P. falciparum sporozoites was used as a control.

After extensive washing in PBS containing 1% BSA, the strips were incubated for two hours at room temperature with affinity-purified ^{125}[I]-labeled goat anti-rabbit IgG. The strips were washed, dried and exposed to autoradiography. The results are shown in Figure 5. The two top bands correspond to the intracellular precursor (67,000 Mr) and membrane (58,000 Mr) forms of the CS protein. Some additional unidentified antigens of lower Mr (probably of mosquito origin[1/]) have been revealed by the antiserum against whole P. falciparum sporozoites (lane 1). Anti-P. falciparum activity was absent in lane 3, as expected).

Example 8: Sporozoite Neutralization by Anti-Conjugate Antisera

The procedure employed was disclosed in Hollingdale, M.R., et al (1984) J. Immunol., 132:909.

1/ The rabbit was immunized with contaminated crude material obtained from the salivary glands of mosquitoes infected with P. falciparum sporozoites.

Immunoglobulin from the serum of one rabbit was purified by chromatography on diethylamino-ethyl cellulose (DEAE-Cellulose) and used in sporozoite in vitro neutralization experiments in accordanced with the Hollingdale procedure: J. Immunol. 132:909(1984). Parasites were obtained from salivary glands of laboratory-bred mosquitoes infected by membrane feeding with cultures of P. falciparum blood stages. Salivary glands were pooled in heat-inactivated human serum, disrupted by trituration and counted. All studies were carried out with human hepatoma (Hep G2-A1G from American Type Culture Collection, Rockville, Md.) cells cultured in Eagle's minimum essential medium (GIBCO, Grand Island, N.Y.) supplemented with 1% human serum.

The results, summarized in Table I, below, show that immune rabbit IgG inhibited parasite development in a dose-dependent manner. Very strong neutralization (between 40-80%) took place even at concentrations of total IgG as low as 2 micrograms/ml (of which not more than 10% is likely to be $(NANP)_3$-specified antibody). When the anti-conjugate antibodies were removed from the IgG by immunoaffinity chromatography using $(NANP)_3$ as the adsorbent, no inhibition in parasite development was observed (Experiment #4). The removal of antipeptide antibodies from the IgG fraction was acertained by immunoradiometric assay in accordance with the method of Example 4.

Table I

Inhibition of *P. falciparum* sporozoite "in vitro" infectivity by antibodies to $(NANP)_3$

Experiment Number	Identification	Number added per culture	Origin	Concentration microgram/ml	EEF*	Percent inhibition
1	7G8	$25x10^3$	pre-immune	200	180	-
			anti$(NANP)_3$	200	26	76
				2	134	26
2	NF54	$17x10^3$	pre-immune	100	203	-
			anti$(NANP)_3$	100	172	15
				20	41	80
				10	21	90
				2	45	78
				1	131	36
				0.1	198	3
3	7G8	$27x10^3$	pre-immune	100	237	-
			anti$(NANP)_3$	100	66	72
				20	11	95
				2	106	55
				1	78	12
				0.1	216	9
4	NF54	$26x10^3$	pre-immune	20	240	-
			anti$(NANP)_3$	20	35	86
				5	76	68
				2	150	38
				0.2	200	17
			anti$(NANP)_3$ adsorbed with $(NANP)_3$	20	228	7
				5	248	0

* Intracellular (exoerythrocytic) forms

Example 9: Recognition of $(NANP)_3$ by Human Antibodies to P.falciparum

To determine whether human antibodies also recognize the repeated epitope of P.falciparum CS protein, the reactivity of such antibodies with $(NANP)_3$ was tested.

Sera from 58 individuals from the Gambia, West Africa (an endemic region) and from 29 healthy blood donors in New York City (not an endemic region) were analyzed by IRMA for the presence of antibodies that would recognize $(NANP)_3$. The assay of Example 4 was employed, except that the second antibody was ^{125}[I] - labeled, affinity purified rabbit anti-human IgG (Sp.act. about 5×10^7cpm/microgram) was used.

To determine the immunoglobulin class the second antibody was used: either ^{125}[I]-labeled, affinity-purified goat anti-human IgG (gamma) or anti-human IgM(mu), both from Kirkegaard & Perry Laboratories, Gaithersburg, MD.

Non-specific binding of antibody to the wells was determined for each individual serum sample by omitting $(NANP)_3$ from the wells. The number of cpm in the control wells was 300-800 (for 1/10 serum dilution). The non-specific cpm was subtracted from the experimental results. The difference (specific binding) is referred to as $\underline{\Delta}$ cpm.

The average Δ cpm of a tenfold dilution of the normal sera was 259 ± 155. This value, plus or minus three standard deviations (724 cpm) was defined as the normal range.

The assay results are plotted in Figure 7.

The percentage of positive sera (Δ cpm > 724) in endemic areas increased with age, ranging from 25% in children (age 1 to 14 years) to 84% in adults over 34 years old. Most positive sera had titers higher than 1/200. The antibody type was IgG.

The specificity of the antisera-$(NANP)_3$ reaction was tested by the inhibition assay of Example 4, i.e. by preincubation of the antisera with a solution of $(NANP)_3$ (50 micrograms/ml). The results are shown in Figure 8.

The antibody-peptide binding was completely inhibited by presence of $(NANP)_3$ in the liquid phase. By contrast, presence of an unrelated synthetic dodecapeptide (corresponding to the repeated epitope of P.knowlesi) failed to inhibit the antisera-$(NANP)_3$ binding.

Example 10: Indirect Immunofluorescence Assay (IFA)

To detect human antibodies directed to the surface membrande of P.falciparum sporozoites an IFA was performed in accordance with the method of Example 5. The purpose was to find the proportion of human antibodies that did not recognize $(NANP)_3$.

Randomly selected IRMA-negative and IRMA-positive sera from individuals older than 20 years and living in a malaria endemic area were tested for antibody specificity to sporozoites either in the presence or in the absence of competing $(NANP)_3$ (50 micrograms/ml). The results are summarized in Table II, below.

TABLE II

Identification of serum	IRMA with $(NANP)_3$ as antigen (Δ cpm)*	IRA with glutaraldehyde fixed sporozoites as antigen	
		Serum Titer*	Serum Titer in the presence of $(NANP)_3$**
G.Z.	9201	4096	320
IDA	4851	1280	<20
8017	3539	640	<20
7930	3501	640	<20
7979	3311	640	<20
7973	2735	320	<20
P-2	2473	320	<20
P-5	2024	320	<20
8012	1765	640	<20
Normal	163	<10	N.D.
7981	168	<10	N.D.
8074	133	20	N.D.
7878	96	<10	N.D.
P-12	75	<10	N.D.
8312	72	<10	N.D.
P-11	-13	<10	N.D.
8286	-91	20	N.D.
7907	-103	<10	N.D.

* When the results of IFA and IRMA were compared by a non-parametric method (Spearman Rank Correlation), the r_s was 0.87 ($p<0.001$). The results of the IRMA in the dilution samples of the positive sera are shown in Figure 2.

** Serum samples were incubated with 50 ug/ml $(NANP)_3$ for 2 hours at room temperature before performing the IFA. N.D. = not done.

The results of the immunoradiometric assay correlated highly (Spearman rank correlation test) with those of the IFA ($p < 0.001$). The presence of $(NANP)_3$ substantially reduced the titers of IRMA-positive sera.

The results of Examples 9 and 10 demonstrate that most human antibodies detected by immunofluorescence (and therefore recognizing only the surface antigen of P.falciparum) were in fact directed against the immunodominant epitope of the P.falciparum CS protein and recognized $(NANP)_3$. These results also highlight the strong immunodominance of the repetitive epitope of the CS protein in man and demonstrate the existence in humans of B-cells that recognize the repeated epitope of the P.falciparum CS protein.

It is expected that these B-cells can be made to respond to a synthetic peptide vaccine either to confer primary immunity or to boost the natural immunity of individuals living in endemic areas.

We claim:

1. A conjugate of an immunogenic peptide said peptide having an amino acid sequence corresponding to that of an immunodominant epitope of P.falciparum circumsporozoite protein and a carrier protein selected from the group consisting of carrier proteins used in vaccine preparations.

2. The conjugate of claim 1 wherein said epitope consists essentially of a tandem repeat of the amino-acid sequence Asn-Ala-Asn-Pro.

3. The conjugate of claim 2 wherein said peptide has been chemically synthesized.

4. The conjugate of claim 3, wherein said peptide has the amino-acid sequence

Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro.

5. The conjugate of claim 1, wherein said carrier protein is selected from the group consisting of diphtheria toxoid, tetanus toxoid, and synthetic random copolymers of amino acids containing lysine or arginine or combinations thereof.

6. The conjugate of claim 4, wherein said protein is tetanus toxoid.

7. A component of a vaccine against the P.falciparum malaria parasite consisting essentially of the conjugate of claim 1.

8. A component of a vaccine against the P.falciparum malaria parasite consisting essentially of the conjugate of claim 2.

9. A component of a vaccine against the P.falciparum malaria parasite consisting essentially of the conjugate of claim 4.

10. A component of a vaccine against malaria consisting essentially of the conjugate of claim 6.

11. The conjugate of claim 4 wherein said epitope consists essentially of a tandem repeat of the amino acid sequence Asn-Ala-Asn-Pro from the C- to the N-terminal.

12. The conjugate of claim 11 wherein said peptide has been chemically synthesized.

13. The conjugate of claim 4 wherein said peptide has the amino acid sequence Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro from the N- to the C-terminal.

14. The conjugate of claim 13 wherein said protein is tetanus toxoid.

15. A component of a vaccine against the P.falciparum malaria parasite consisting essentially of the conjugate of claim 11.

16. A component of a vaccine against the P.falciparum malaria parasite consisting essentially of the conjugate of claim 13.

17. A component of the vaccine against malaria consisting essentially of the conjugate of claim 14.

18. The conjugate of claim 11 wherein said peptide has the sequence Cys-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro from the N-terminal to the C-terminal.

19. The conjugate of claim 18 wherein said peptide has been chemically synthesized.

20. The conjugate of claim 11 wherein said peptide has the amino acid sequence Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Cys from the C-terminal to the N-terminal.

21. The conjugate of claim 20 wherein said peptide is chemically synthesized.

22. The conjugate of claim 18 wherein said peptide is tetanus toxoid.

23. The conjugate of claim 20 wherein said peptide is tetanus toxoid.

24. A component of the vaccine against the P.falciparum malaria parasite consisting essentially of the conjugate of claim 18.

25. A component of the vaccine against the P.falciparum malaria parasite consisting essentially of the conjugate of claim 20.

26. A vaccine against the sporozoite stage of the P.falciparum malaria parasite comprising a conjugate of a peptide having the sequence Cys-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro,

a carrier protein and a vaccine adjuvant, said vaccine suitable for immunizing mammals.

FIG. 1

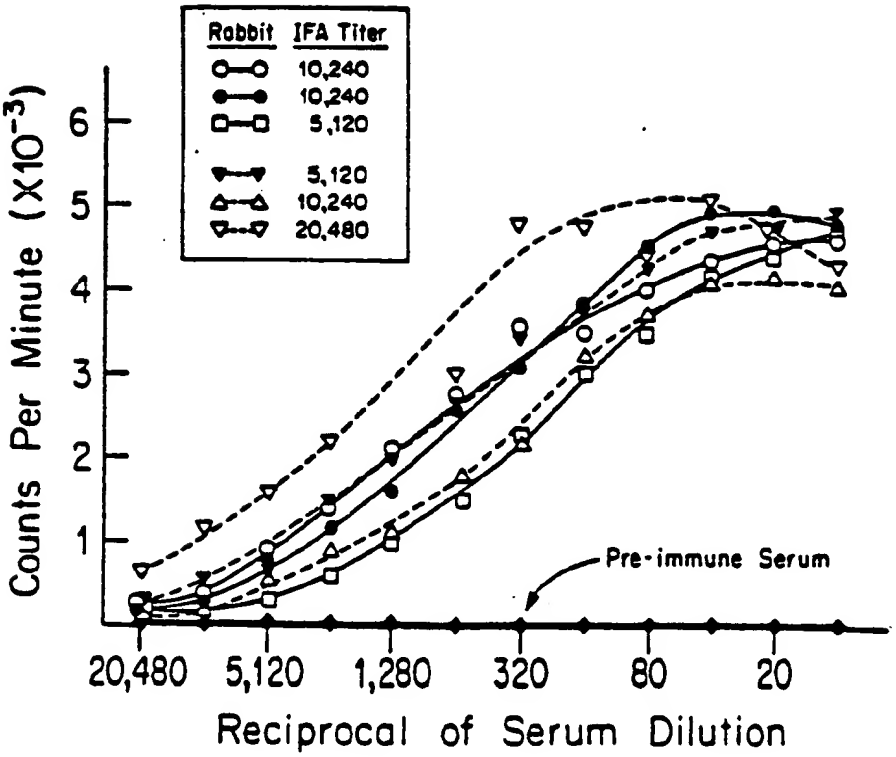

Rabbit IFA Titer
10,240
10,240
5,120
5,120
10,240
20,480
Counts Per Minute (X10⁻³)
1
2
3
4
5
6
Pre-immune Serum
20,480
5,120
1,280
320
80
20
Reciprocal of Serum Dilution

FIG. 2

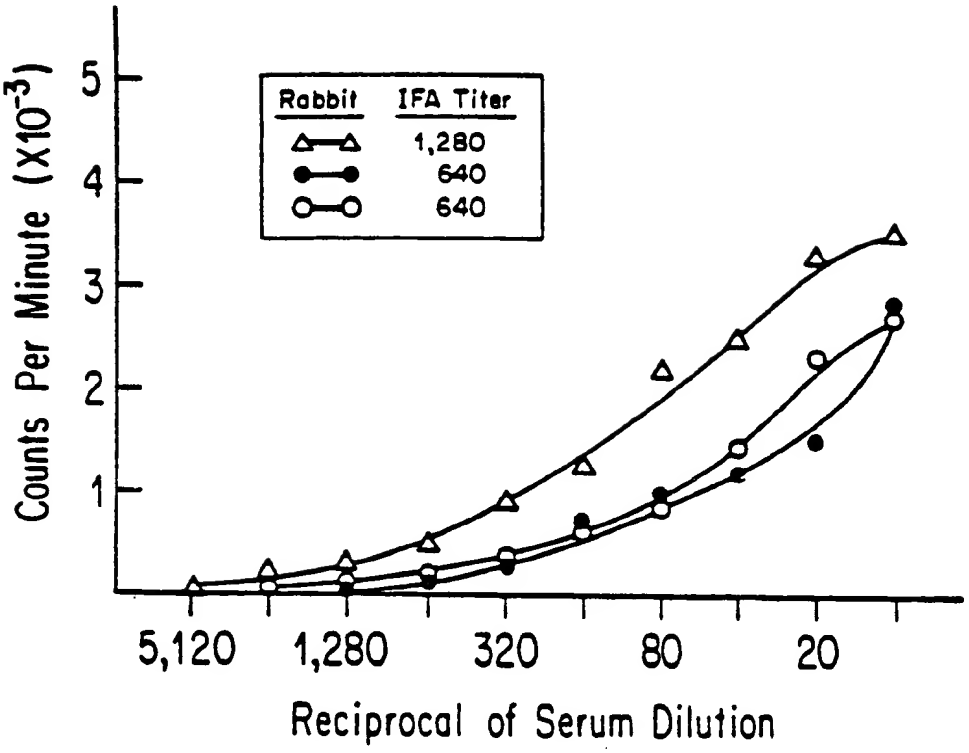

Counts Per Minute (X10^{-3})
5
4
3
2
1
Rabbit
IFA Titer
1,280
640
640
5,120
1,280
320
80
20
Reciprocal of Serum Dilution

FIG. 3

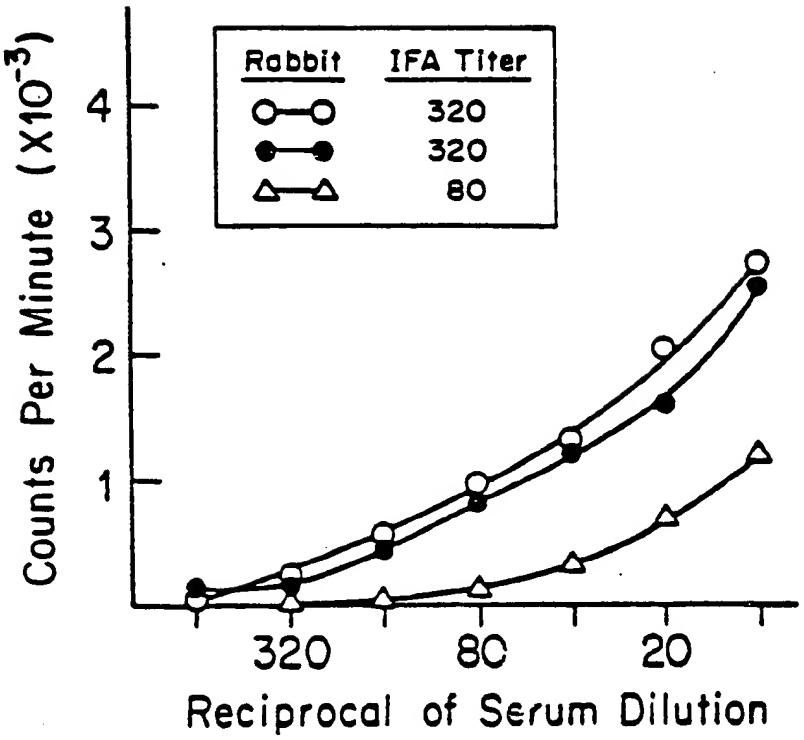

Rabbit
IFA Titer
320
320
80
Counts Per Minute (X10⁻³)
4
3
2
1
320
80
20
Reciprocal of Serum Dilution

FIG. 4

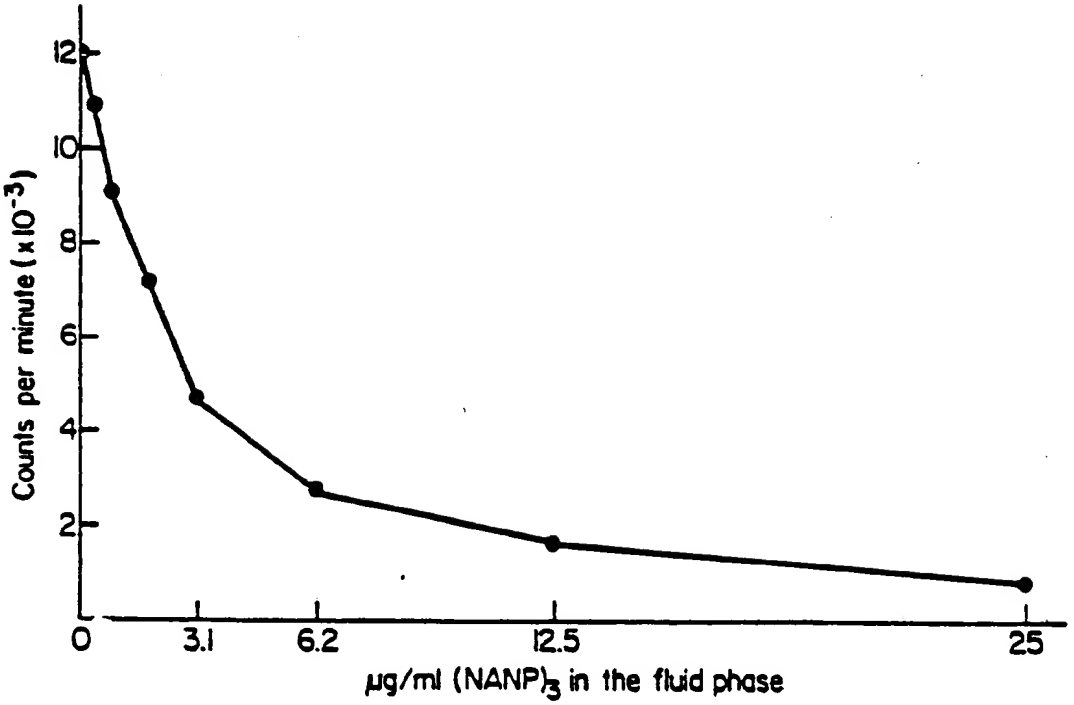

Counts per minute (x10⁻³)
12
10
8
6
4
2
0
3.1
6.2
12.5
25
µg/ml (NANP)3 in the fluid phase

FIG. 5

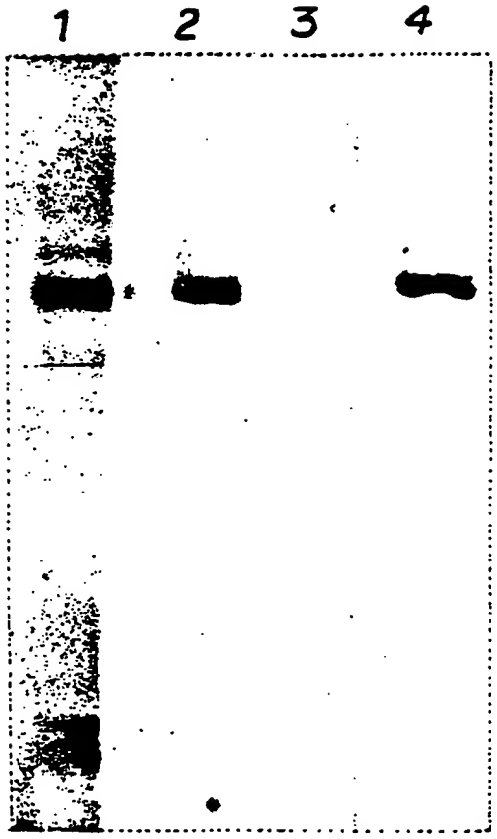


FIG. 6

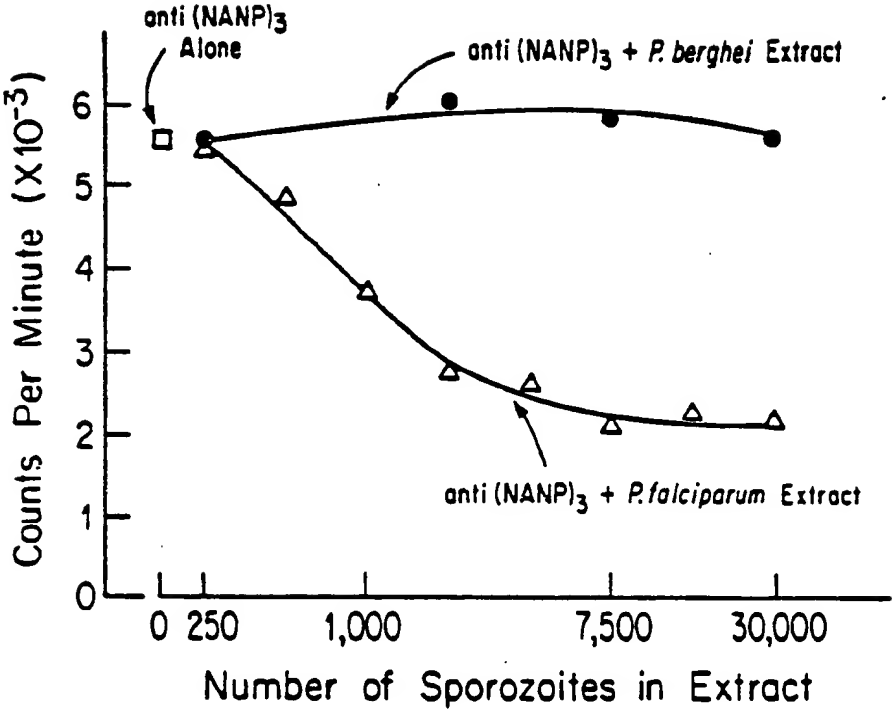

anti (NANP)3
Alone
anti (NANP)3 + P. berghei Extract
anti (NANP)3 + P. falciparum Extract
Counts Per Minute (X10-3)
0
1
2
3
4
5
6
0 250
1,000
7,500
30,000
Number of Sporozoites in Extract

FIG. 7

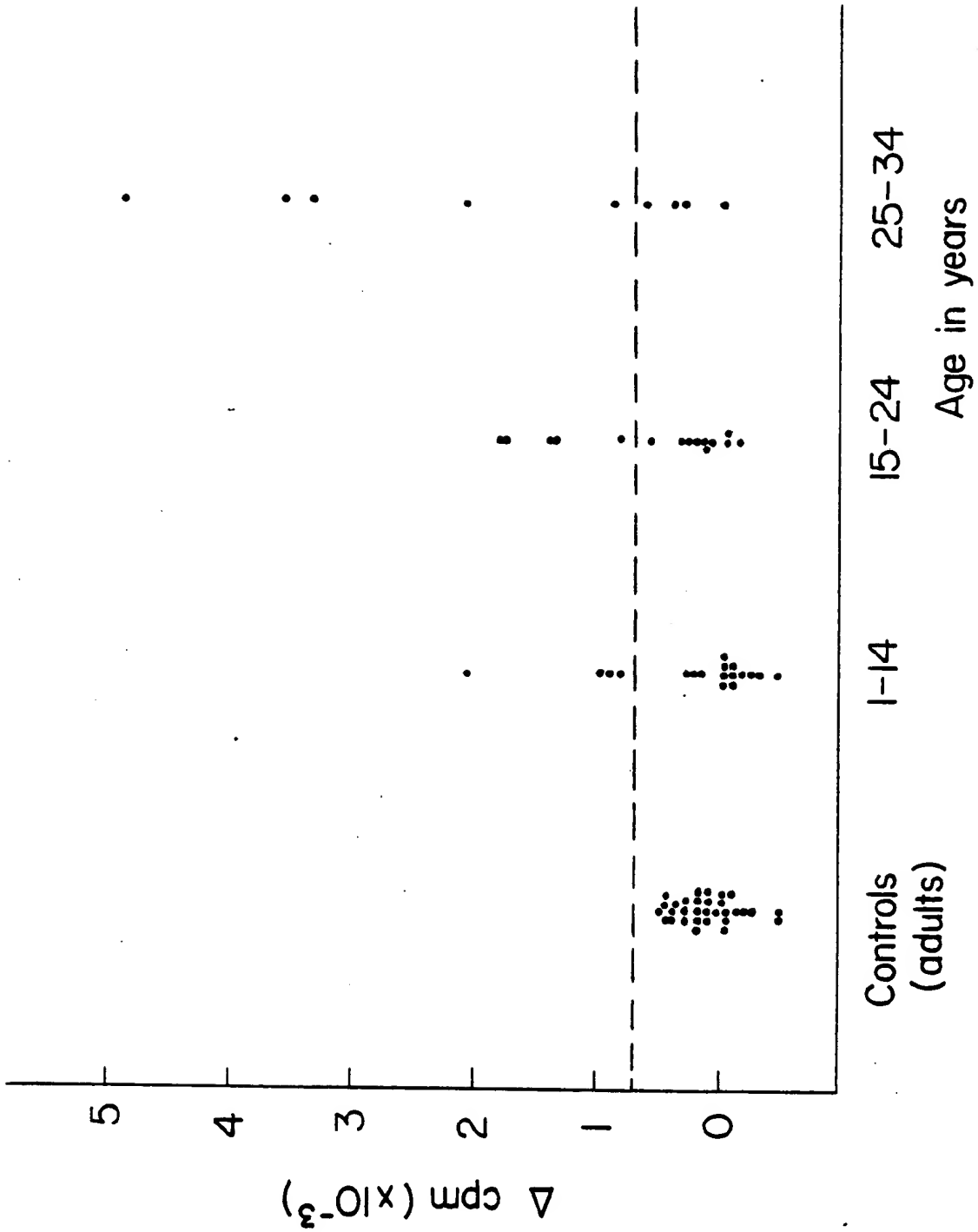
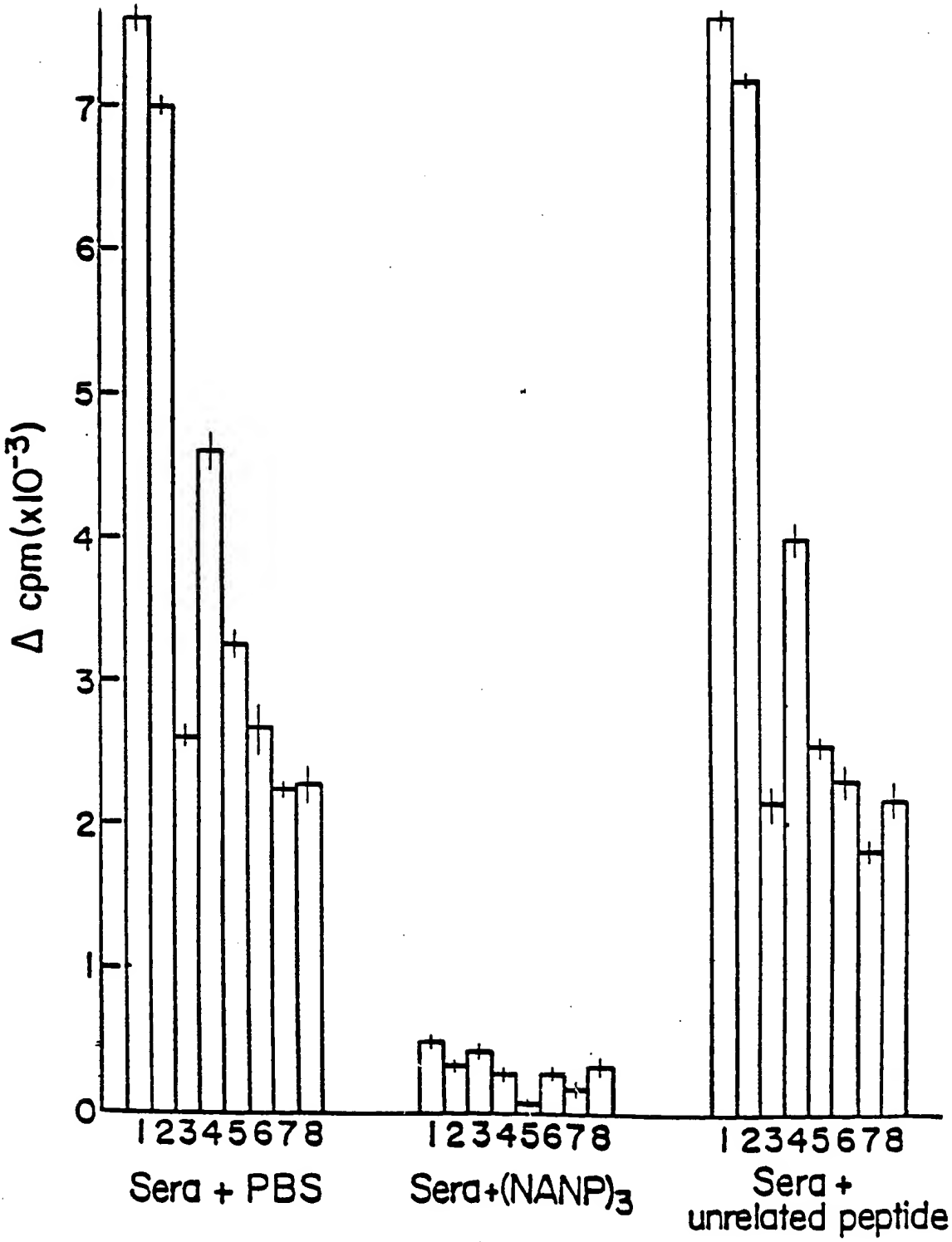


FIG. 8


Δ cpm (x10⁻³)
0
1
2
3
4
5
6
7
1 2 3 4 5 6 7 8
Sera + PBS
1 2 3 4 5 6 7 8
Sera+(NANP)3
1 2 3 4 5 6 7 8
Sera + unrelated peptide

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/00627

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4) C07K 5/00, 7/08, 15/12, 17/00; A61K 37/02, 39/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	530/300, 327, 330, 806, 810; 424/88, 89; 514/2, 895

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Computer search of Databases: Georgetown, STN CA
1) Asn-Ala-Asn-Pro 2) Plasmedium Falciparum and (conjugate/link) and toxin.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y,P	US,A, 4,554,101 (HOPP) Published 09 NOVEMBER 1985	1-26
Y	N, Cell, Vol. 37, Issued 1984, Wilson et al, "The Structure of an Antigenic Determinant in a Protein", pages 767-78.	1-26
Y	N, Science, Vol. 225, Issued 1984, Dame et al, "The Structure o f the Gene Encoding the Immunodominant Surface Antigen on the Sporozoite of the Human Malaria Parasite Plasmodium Falciparum", pages 593 to 599.	1-26
Y	N, Science, Vol. 225, Issued 1984, Enea et al, "DNA Cloning of Plasmodium Falciparum Circumsporozoite Gene: Amino Acid Sequence of Repetitive Epitope, pages 628-629.	1-26
Y	N, Exp. Med., Vol. 153, Issued 1983, Zavala et al, "Circumsporozoite Protein of Malaria Parasites Contain Identical Epitopes", pages 1947-57.	1-26
Y,P	N, Science, Bol. 228, Issued 1985, Zavala et al, "Rationale for De elopment of Synthetic Vaccines Against Plasmodium falciprum Malaria", Pgs. 1436-40.	1-26

* Special categories of cited documents: [13]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [9]
28 MAY 1986	20 JUN 1986
International Searching Authority [1]	**Signature of Authorized Officer [10]**
ISA/US	Garnette D. Draper GARNETTE D. DRAPER

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	N, Molecular and Biochemical Parasitology, Vol. 5, Issued 1982, Goman et al, "The Establishment of Genomic DNA Libraries for the Human Malaria Parasite Plasmodium Falciprum and Identification of Individual Clones by Hybridization" pages 391-97.	1-26

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.